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Increased mobility and stem-cell proliferation rate in *Dugesia tigrina* induced by 880 nm light emitting diode

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ABSTRACT

The therapeutic effects elicited by photobiostimulation in the near infrared range may be associated with increased proliferation rate of particular cell-types. The present study utilized commercial light emitting diodes to investigate the effects of low-level near-infrared radiation on the proliferation rate of stem cells in amputated planarian. Whole and amputated animals were exposed to either ambient diurnal lighting, darkness, white light, red light, or near-infrared (880 nm) light. Irradiation was consistent for the duration of the experiments and was provided using commercial 5 mm light emitting diodes (~1.0 mW/m^2 in power density and ~0.01 J/cm^2 in radiant exposure). Compared to other groups amputated planarian exposed to near-infrared displayed increased mobility by the 3rd day of exposure ($F_{(4,26)} = 4.31$, $p < 0.04$, $\eta^2 = 41\%$). Higher densities of stem cells were measured in these worms 84 h post injury ($F_{(4,72)} = 4.78$, $p < 0.01$, $\eta^2 = 21\%$). These findings suggest that non-coherent light sources with power-densities about 1000 times lower than contemporary low-power laser settings remain effective in generating photobiostimulation effects and warrants further investigation on stem-cell proliferation induced by near-infrared light emitting diodes.

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1. Introduction

The effects of low power near-infrared (NIR) radiation on biological systems have been studied for at least 40 years [1]. NIR radiation in the 650–900 nm range can initiate a variety of therapeutic effects, such as acceleration in wound healing [2,3], fibroblast proliferation [3,4], and collagen synthesis [2,5]. Recent studies following the development of light emitting diodes (LEDs) showed comparable effects can be elicited by non-coherent LED sources. The most reliable responses have been the prevention of oral mucositis and attenuation of experimentally-induced optic nerve degeneration [6–8].

NIR irradiation results in a general increase in available pools of energy. The correlative stimulation of mitochondrial production of ATP [9,10] allows the irradiated tissue or cell type to accelerate specific functions [11]. Wounds produce collagen faster, irradiated embryonic fibroblasts display mature fibroblast locomotory characteristics [2,12], and irradiated cancer cell lines proliferate at higher rates when exposed to the NIR [12,13]. However, *in vivo* studies with porcine and rat subjects have been inconsistent [14,15].

To examine the effects of NIR radiation on biological systems *in vivo*, we selected the fresh water Turbellarian planarian. Planarians are known for their remarkable capability to regenerate lost body parts. This regenerative process is dominated, in its initial stage, by the proliferation of the only actively mitotic planarian cell, the pluripotent neoblast [8,16,17]. Planaria show changes in behaviour and regenerative activity in response to NIR [18]. The present study was designed to discern the effects of low power, non-coherent, 880 nm NIR radiation on regenerating planarian behaviour and stem-cell proliferation *in vivo*. Regenerating planaria will act as a proliferation-biased model of wound healing as well as provide a simplified *in vivo* platform for NIR irradiation.

2. Methods

Planarian *Dugesia tigrina* were obtained from Carolina Biology Supplies (Burlington, North Carolina) and maintained for 2 months prior to the start of experimentation to ensure adaptation and habituation to the laboratory setting. The planarians were housed in 500 ml glass containers refilled with aerated spring water (President's Choice, Brampton, Ontario) after weekly, 1-h feedings of approximately 2 g of frozen bovine liver. Ambient diurnal

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fluorescent lighting was approximately 12:12. Water temperature remained between 19 °C and 21 °C for the extent of the experiment. Prior to experimentation, planarians were starved for 4–6 days to avoid contamination due to digestion of foods.

Adult worms between 1 and 2 cm in length were selected for experimentation. The animals were placed on a block of ice for 30 s for immobilization and anaesthesia prior to cutting with a surgical blade. Cuts were made in the transverse plane just posterior to the auricles. The injured posterior fragment was subjected to the study. Specimens under experimentation were kept in 20 ml glass containers filled with aerated spring water and subject to one of five experimental conditions.

The conditions consisted of exposures to either: (1) ambient diurnal lighting, (2) white light, (3) red light, (4) NIR light, or darkness for the entire duration of the experiment. Except for the fluorescent ambient condition (normal housing), planarians were housed in a compartmentalized dark box with arrays of 3 LEDs per lighting condition (100 mA, 1.5 V bulbs, Perkin Elmer Optoelectronics, Salem, Massachusetts). Each of the three diodes operated at 65 mW for a total output of 195 mW per lighting condition. White light was produced using indium–gallium/yttrium–aluminum–garnet (InGa/YAG) diodes, red light was produced using gallium–arsenide–phosphorous/gallium–phosphorous (GaAsP/GaP) 630 nm diodes, and NIR was produced using gallium–aluminum–arsenide (GaAlAs) 880 nm diodes. The LED arrays were placed 1 cm above the experimental glass containers. The diameters of the experimental containers were 5 cm. The total power densities for the LED conditions were about 1.2 mW/m^2 as measured by both a luxmeter and a photomultiplier tube. On the basis of results of a pilot study and the relative low power density of LED lights (compared to the typical 900 W/m^2 output of non-thermal lasers employed in NIR biostimulation studies [5]), we reasoned that a constant 24 h/day exposure paradigm would not be excessive for the vitality of the worms.

2.1. Planarian mobility

Thirty amputated worms were measured for mobility once every 24 h post injury. Planarian mobility was tested in a 22 cm by 22 cm glass dish filled with aerated spring water to a depth of 1.5 cm. A single white InGa/YAG LED was placed at one corner of the dish with the light beam pointed to the bottom of the dish to motivate the photophobic planarian to occupy a darkened area. Specimens to be tested were placed into the dish 5 cm from both of the edges closest to the white light and observed for 1 min. The displacement, distance travelled, and final location of each specimen after the 1-min period were recorded in cm. The displacement and final location data allowed the experimenter to infer restoration of classic planarian negative phototaxis, while distance travelled was used to infer general mobility and activity in a novel open field setting.

2.2. Neoblast count

Seventy-five amputated worms were examined histologically by light microscopy. The specimens were harvested after 84 h of treatment, between days 3 and 4 post injury. The specimens were fixed in cold ethanol–formaldehyde–acetic acid solution (72 part anhydrous ethyl alcohol, 18 part distilled H20, five part 37% formaldehyde, five part glacial acetic acid), processed and embedded in paraffin wax. Whole planaria were sectioned along the frontal plane at 2 μm. The slices were then stained with Harris' Haematoxylin and Eosin and visualized under a light microscope [18]. Four separate 10,000 μm^2 fields within the regeneration blastema for each specimen were identified and the neoblasts within these fields quantified. The mean value of these four counts was used to infer the density of neoblasts per 10,000 μm^2 per specimen.

2.3. Statistics

One-way between subject analysis of variance (ANOVA) was used to analyze the data collected. The significance level was set at 0.05. Tukey's post hoc test was used to analyze significant one-way ANOVA results.

3. Results

The mobility of regenerating planarian ranged from approximately 4 cm on day 1 to approximately 8 cm on day 7 post injury. By day 4, the mean distance travelled by regenerating planarians was significantly higher (at 6 cm) than on previous days ($F_{(6, N=210)} = 31.78$, $p < 0.01$, $\eta^2 = 48\%$). The increased mobility had achieved asymptote by day 5 (Fig. 1). There was also a significant shift in the displacement and final localization of the regenerating planarians over time (Chi-Square$_{(6, N=210)} = 99.84$, $p < 0.01$, Pearson $R = 0.57$). Prior to day 4, regenerating planarians were not sensitive to light and remained around the drop-off point. By day 5, regenerating planarians consistently localized away from the light source (Fig. 2). The displacement and localization was not significantly different across treatment conditions.

The only significant difference found between conditions was mobility on day 3. NIR irradiated specimens travelled (Fig. 3) significantly further within a 1-min testing period than those exposed to either the white light or complete darkness ($F_{(4,26)} = 4.31$ $p < 0.01$ $\eta^2 = 41\%$). However there were no statistically significant differences between the NIR and red light groups. Although pilot studies suggested that whole planarians displayed increased mobility under NIR irradiation compared to all other groups, this effect was not seen in regenerating specimens after the 3rd day.

The noticeable inflection point in measured mobility and displacement at day 4 of the planarian's regeneration process determined the time of our histological examination of the specimens 84 h post injury. One way analysis of variance indicated that NIR exposed specimens contained significantly higher numbers of neoblast per unit area in the regenerating blastema (Fig. 4) compared to those that had been exposed to other treatments ($F_{(4,72)} = 4.78$, $p < 0.01$, $\eta^2 = 21\%$). The approximately 35% increase in neoblast proliferation 84 h post injury for the NIR-exposed planaria were qualitatively apparent under light-microscopy (Fig. 5).

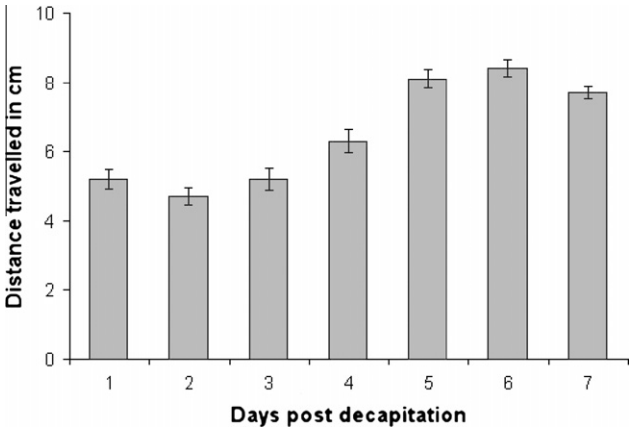

Fig. 1. Bar graph plot of distance travelled by regenerating planaria across 7 days following amputation. Significant increase in mobility was observed on day 4 ($F_{(6, N=210)} = 31.78$, $p < 0.01$, $\eta^2 = 48\%$) and leveled off by day 5. Error bars represent standard errors of the mean.

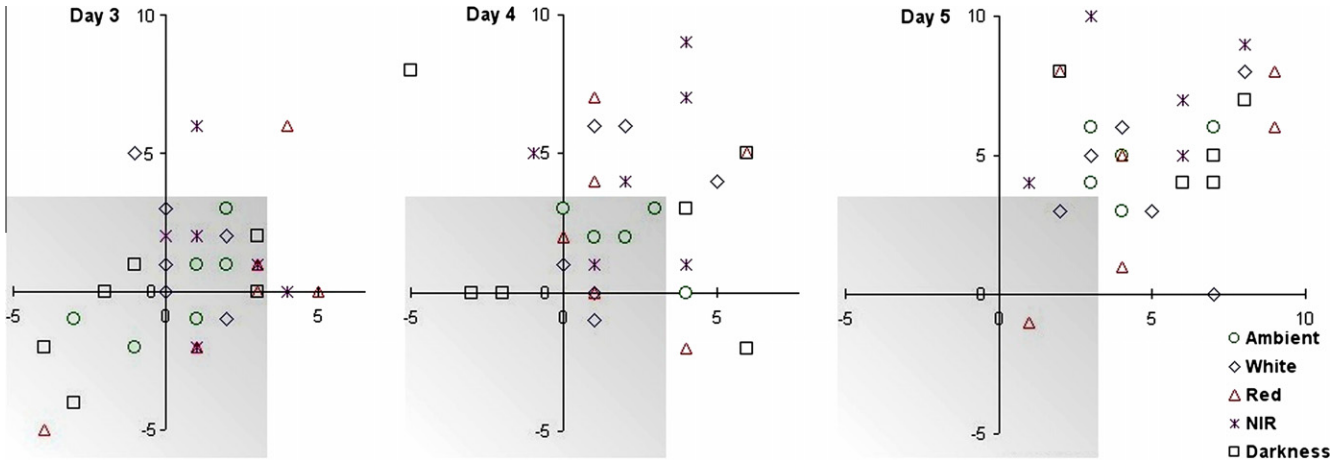

Fig. 2. Planarian displacement on days 3, 4, and 5 post injury. Co-ordinates are in centimetres. Light source is located at co-ordinate (−5, −5) and drop-off point at (0, 0). Shaded area represents area of planarian localization considered to be insensitive to light. There was a significant shift in sensitivity to light across time (Chi-Square$_{(6,\ N=210)}$ = 99.84, $p < 0.01$, Pearson $R = 0.57$).

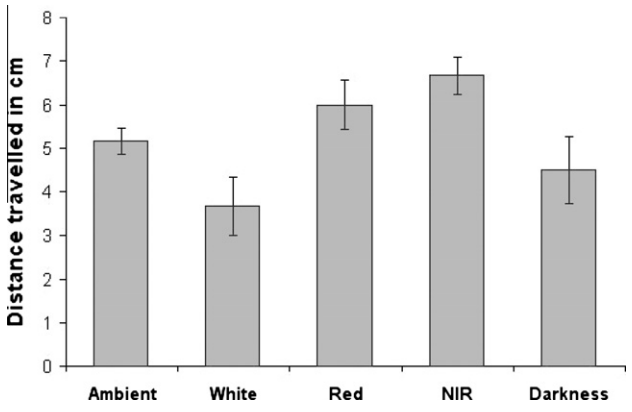

Fig. 3. Planarian mobility on day 3 post injury. Near infrared irradiated specimens travelled significantly further than white light irradiated specimens ($F_{(4,26)} = 4.31$, $p < 0.04$, $\eta^2 = 41\%$).

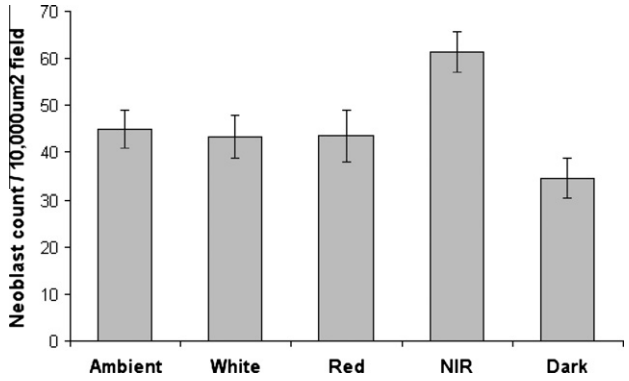

Fig. 4. Neoblast count in the regenerating blastema 84 h post injury. Near infrared irradiated specimens displayed an increase in neoblast density within 10,000 μm^2 fields sampled ($F_{(4,72)} = 4.78$, $p < 0.01$, $\eta^2 = 21\%$).

4. Discussion

The present study indicated that neoblast proliferation [18] can also be reproduced by using non-coherent light NIR (880 nm) sources with power-densities about a 1000-fold less than conventional low-power lasers. This supports the growing number of studies that shows LEDs have photobiostimulation effects comparable to lasers [19–21]. Contemporary photobiostimulation studies using low-power lasers or LEDs have demonstrated numerous effects associated with increased cellular proliferation, maturation, and activity [2,4,10]. These effects have been most consistently observed for fibroblast and osteoblast cultures [4,22] and have been attributed to an increase in mitochondrial activity following light stimulation in the near infrared range [9,10,23]. Although *in vitro* experiments have proved fruitful for both demonstrating photobiostimulatory effects and elucidating its cellular mechanisms, *in vivo* mammalian studies have remained relatively sparse and contradictory.

Negative results from *in vivo* studies may be attributed to differences in "tuning" (resonance matching) of the radiation, the relative attenuation of externally applied NIR photons within deep structures of large volume organisms, or the greater complexity of whole animal systems. Planaria may be an optimal animal model to study the effects of NIR photobiostimulation on intact animals [18] because of their relative "transparency" and the rate of rapid cell division that in most cell systems enhances sensitivity to subtle levels of incident energies [24,25] and results in qualitatively different biological consequences. The present study revealed that low power NIR radiation induced increases in planarian mobility and neoblast proliferation on the 3rd day of regeneration. The non-distinction between the effects of red light and NIR radiation on planarian mobility further illustrates the complexity involved with measuring photobiostimulation effects at the whole animal level. However, with simple, measurable behaviour and only one actively mitotic and pluripotent cell type that is sensitive to NIR irradiation, the planarian provides a simple whole organism platform to fine tune photobiostimulation specifics and isolate the potential biophysical mechanisms of the process.

The biophysical mechanisms to explain the specific effect by the NIR energies upon cell proliferation could involve the convergence of magnitudes at the level of the membrane. With the NIR power of about 10^{-3} W/m^2 employed in our study the energy contained within a symmetrical cross-section of membrane (10 nm in width) would be in the order of 10^{-19} J. The energy associated with the 880 nm wavelength (3.4×10^{14} Hz) when multiplied by Planck's constant is 2.2×10^{-19} J. This convergent value suggests a potential resonance phenomenon and is within a factor of 10 of the quantum of 2×10^{-20} J associated with maintaining the membrane potential and the interactions of adhesive and cohesive processes [26] necessary for cell vitality and division. Variations of intrinsic

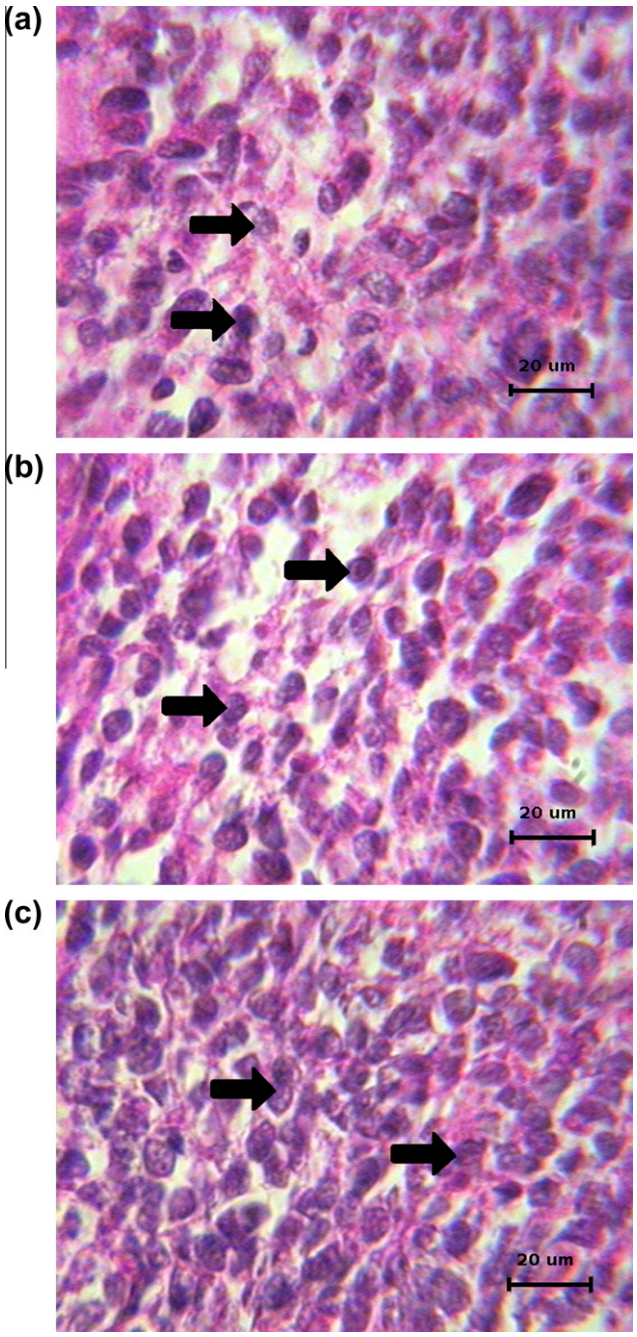

Fig. 5. Sample histological section of planarian wound blastema 84 h post injury for the white light irradiated (a), darkness (b), and near infrared irradiated groups (c). Near infrared irradiated sections displayed an increase in number of neoblasts (arrow). Sections were cut at 2 μm and stained Haematoxylin and Eosin (Bar = 20 μm).

mechanical vibrations or extremely low frequency electromagnetic fields around 10 Hz from the organism itself could produce the required congruency to match both coefficients and order of magnitude for this quantum.

There is also Cosic's [27] classic and seminal model that has been under-applied in biophysical phenomena. She predicted that macromolecular protein activity and protein absorbance of electromagnetic radiation can be predicted *de novo* through a protein's inherent resonance characteristics. Her resonant recognition model of protein characterization involves the representation of amino acid sequences as a numerical series of each amino acid's electrical

Table 1
Area of regenerating planarian blastema across time.

Treatment condition	Area of regenerating blastema post injury (mm^2)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Ambient	0.046	0.077	0.204	0.420	0.693	0.713	0.806
White	0.052	0.052	0.190	0.360	0.587	0.703	0.793
Red	0.037	0.060	0.159	0.415	0.638	0.661	0.728
NIR	0.032	0.078	0.165	0.409	0.629	0.652	0.703
Dark	0.037	0.070	0.168	0.441	0.575	0.588	0.721
Average change compared to previous day		+0.027	+0.120	+0.232	+0.215	+0.039	+0.087

No significance across treatment conditions were found. $N_{(condition)} = 6$.

pseudo potential. It is derived from the energy of delocalized electrons within each amino acid. The numerical series of electrical pseudo potentials can be visualized in the frequency domain through Fourier transform. Characteristic frequencies revealed through the resonance model would then represent recurring spatial orientations of specific electrical potentials within the protein structure.

Application of Cosic's procedure of cross spectral comparisons to cytochrome C, an important contributor to mitochondrial function, and cytochrome C oxidase II proteins, revealed a significant peak of interaction. For activation of cytochrome C proteins by laser stimulation, peak spectral frequencies are .47 and .36 (using her formula) which correspond to 498 and 650 nm, respectively. However the peak spectral frequency for the interaction between cytochrome C and cytochrome oxidase II displayed a large amplitude peak around .27 which corresponds to 867 nm and is within the range (considering intrinsic variability of measurement and molecular forms) to 880 nm.

This study and pilot studies before also measured the area of regenerating planarian blastema (Table 1), the results of which attest to well established speed of planarian regeneration. Although the remarkable speed of planarian regeneration likely masked NIR treated effects at the behavioural and whole organism level, the present study and others [18] have shown that low-power photobiostimulation can increase planarian stem-cell proliferation approximately 4 days after initial injury. NIR radiation may affect the proliferative blastema formation stage of planarian regeneration and target the cell-type undergoing high mitotic activity. Such a process would be analogous to fibroblast proliferation seen in the proliferative stage of wound healing. Further studies focused on the molecular biology of neoblasts are warranted for generalization of NIR radiation-induced stem-cell proliferation in human cell cultures. The beneficial effects of NIR irradiation on biological systems may be dependent upon the properties of the cells rather than an intrinsic property that produces ubiquitous health or salubrity.

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